16:32:37 OCA PAD INITIATION - PROJECT HEADER INFORMATION 08/22/91

Active

Project #: E-19-660 Cost share #: Rev #: 0
Center # : 10/24-6-R7254-0A0 Center shr #: OCA file #:
Work type : RES
Contract#: 1 R29 HL44960-01A1 Mod #: Document : GRANT
Prime #: Contract entity: GTRC

Subprojects ? : N CFDA:
Main project #: PE #: N/A

Project unit: CHEM ENGR Unit code: 02.010.114
Project director(s):
WICK T M CHEM ENGR (404)894-8795

Sponsor/division names: DHHS/PHS/NIH / NATL INSTITUTES OF HEALTH
Sponsor/division codes: 108 / 001

Award period: 910725 to 920630 (performance) 920930 (reports)

Sponsor amount	New this change	Total to date
Contract value	112,313.00	112,313.00
Funded	112,313.00	112,313.00
Cost sharing amount		0.00

Does subcontracting plan apply ?: N

Title: THE MECHANISM OF SICKLE ERYTHROCYTE/ENDOTHELIAL ADHESION

PROJECT ADMINISTRATION DATA

OCA contact: Kathleen R. Ehlinger 894-4820

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BETHESDA, MD 20892

Security class (U,C,S,TS) : U ONR resident rep. is ACO (Y/N): N
Defense priority rating : N/A NIH supplemental sheet
Equipment title vests with: Sponsor GIT X

Administrative comments -
INITIATION OF PROJECT. YEAR 1 OF 5 YEAR "FIRST AWARD".

GEORGIA INSTITUTE OF TECHNOLOGY
OFFICE OF CONTRACT ADMINISTRATION

NOTICE OF PROJECT CLOSEOUT

Closeout Notice Date 08/21/92

Project No. E-19-660 Center No. 10/24-6-R7254-0A0

Project Director WICK T M School/Lab CHEM ENGR

Sponsor DHHS/PHS/NIH/NATL INSTITUTES OF HEALTH

Contract/Grant No. 1 R29 HL44960-01A1 Contract Entity GTRC

Prime Contract No.

Title THE MECHANISM OF SICKLE ERYTHROCYTE/ENDOTHELIAL ADHESION

Effective Completion Date 920630 (Performance) 920930 (Reports)

Closeout Actions Required:	Y/N	Date Submitted
Final Invoice or Copy of Final Invoice	N	
Final Report of Inventions and/or Subcontracts	N	
Government Property Inventory & Related Certificate	N	
Classified Material Certificate	N	
Release and Assignment	N	
Other	N	

CommentsCONTINUED BY E-19-X04; CLOSING DOCUMENTS WILL BE ISSUED AT THE "END" OF THE GRANT.

Subproject Under Main Project No.

Continues Project No.

Distribution Required:

Project Director	Y
Administrative Network Representative	Y
GTRI Accounting/Grants and Contracts	Y
Procurement/Supply Services	Y
Research Property Managment	Y
Research Security Services	N
Reports Coordinator (OCA)	Y
GTRC	Y
Project File	Y
Other	N
	N

E-19-660

PROGRESS REPORT SUMMARY	GRANT NUMBER HL44960-02	
PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR Timothy M. Wick	PERIOD COVERED BY THIS REPORT FROM	THROUGH
APPLICANT ORGANIZATION Georgia Institute of Technology	07/25/91	06/30/92

TITLE OF PROJECT *(Repeat title shown in item 1 on first page)*
Mechanism of Sickle Erythrocyte/Endothelial Cell Adhesion
(SEE INSTRUCTIONS)

SR.946

1. Specific Aims

A prominent factor contributing to the morbidity and mortality of sickle cell anemia is microvascular occlusion by sickle red blood cells. It has been suggested that young sickle red cells adhere to microvascular endothelium and delay transit of subsequent cells. This partial impediment selectively traps the old, dense, mechanically rigid sickle cells and irreversibly sickled cells; leading to complete occlusion, ischemia, and tissue damage.

Sickle erythrocytes are abnormally adhesive to endothelial cells *in vitro*. The level of *in vitro* adhesion quantitatively correlates with the clinical severity of sickle cell disease, suggesting that adherence may be a determining factor in the clinical variability reported in patients. Adhesion likely occurs in the post-capillary venules, where red cells are in close proximity to the endothelium and blood-shearing forces opposing adhesion are low.

We propose to elucidate the mechanism(s) of sickle cell adherence to endothelial cells under physiologically relevant flow conditions *in vitro*. This will be accomplished through studies defined by the following specific aims:

1. Characterize the difference between sickle cell adherence to large-vessel and microvascular endothelium by determining the effects of agonists and antagonists on adherence.
2. Identify plasma factors, including factors released from activated sickle platelets, responsible for adherence.
3. Determine the variability in sickle cell adhesion to endothelial cells from patient to patient and for individual patients during crisis and asymptomatic periods.
4. Contrast the effects of agonists which presumably stimulate endothelial cells, such as thrombin, endotoxin, plasmin, histamine, fibrin(ogen), interleukin-1, and tumor necrosis factor on adherence.

2. Studies and Results

Methods

Adherence of washed sickle red blood cells suspended in various media (e.g. plasma, serum-free medium, serum-free medium containing purified proteins) to human umbilical vein (1) or human dermal microvascular endothelial cell (2) monolayers is measured at a shear stress of 1.0 dyne/cm^2 essentially as described in the original application. Present studies are confined to asymptomatic patients to determine which parameters are important in adherence.

Differences between adherence to umbilical vein and microvascular endothelium.

We have previously reported that high molecular weight von Willebrand factor (vWF) multimers promote sickle, but not normal, red cell adhesion to umbilical vein endothelium (1). However, high molecular weight vWF multimers only promote low levels of sickle red cell adherence to microvascular endothelial cells (Figure 1). In contrast, autologous sickle plasma promotes greater sickle cell adherence to microvascular as compared to umbilical vein endothelium (Figure 2). Additional studies on 45 patients indicate that plasma-mediated sickle cell adherence to microvascular endothelial cells varies over three orders of magnitude (data not shown).

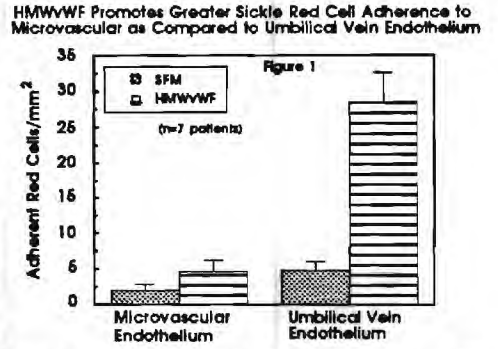


Figure 1. Washed sickle cells were suspended in serum-free medium (SFM) or SFM containing high molecular weight vWF (HMWvWF) and adherence to microvascular and umbilical vein endothelium was measured at a shear stress of 1.0 dyne/cm^2.

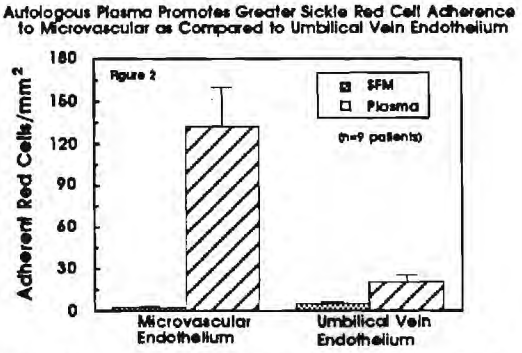


Figure 2. Washed sickle cells were suspended in SFM or SFM containing 30% autologous sickle plasma (Plasma).

These data suggest that the mechanism of sickle cell adherence to large vessel and microvascular endothelium is qualitatively different. Since adherence is likely of clinical significance in the microcirculation, we have undertaken additional studies to further characterize plasma-mediated sickle cells adherence to microvascular endothelium.

Plasma factors which promote adherence

Additional data indicate that the adhesion promoting components of sickle plasma are greater than 100 kDa molecular weight (3 patients, not shown) and that collagen-binding proteins account for 56% of the plasma mediated adherence (11 patients, not shown), suggesting that large adhesive proteins (vWF [3], thrombospondin [4], fibronectin [5] all bind to collagen) in sickle plasma account for the observed adherence.

Activated Sickle Platelet Supernatant Promotes Sickle Cell Adherence

More recently, we have become interested in the role of activated platelets in sickle cell adherence. To test the hypothesis that activated sickle platelets secrete factors which promote sickle cell adherence, we isolated sickle platelets from platelet-rich plasma and activated them with 0.1U/ml thrombin in SFM (6). The activated sickle platelet supernatant (ASPS) was collected and used as a red cell suspending medium. ASPS promoted high levels of sickle, but only moderate levels of normal red cell adherence to microvascular endothelium (Figure 3). Preincubation of microvascular endothelium with an antibody (OKM5) against the thrombospondin receptor (CD36) (7) inhibits 86% of the ASPS-mediated adherence (Figure 4).

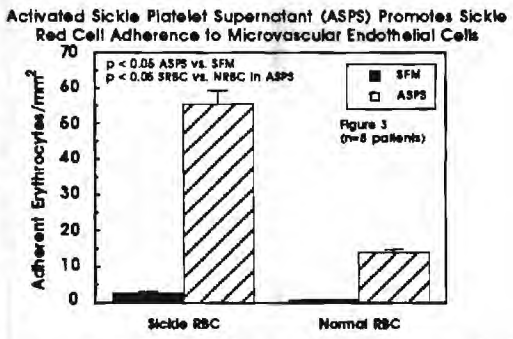


Figure 3. Sickle platelets were isolated, washed, and activated for 10-min with 0.1U/ml thrombin in a volume of SFM equal to the plasma volume. Sickle or normal red cells were suspended in ASPS and adherence to microvascular endothelium was measured as described.

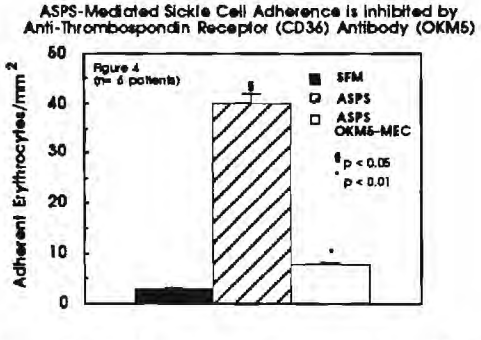


Figure 4. Sickle cells were suspended in ASPS and adherence to endothelium preincubated with anti-CD36 antibody (OKM5) was compared to antibody-free endothelium.

Thrombospondin is stored in platelet α-granules and released upon platelet activation (8). The data of Figure 4 indirectly implicate thrombospondin as a factor in ASPS which mediates adherence. As shown in Figure 5, purified thrombospondin in SFM promotes 6-fold greater sickle as compared to normal red cell adherence to microvascular endothelium (at a plasma concentration of thrombospondin). When the suspension concentration of thrombospondin is doubled, adherence increases proportionally (Figure 6).

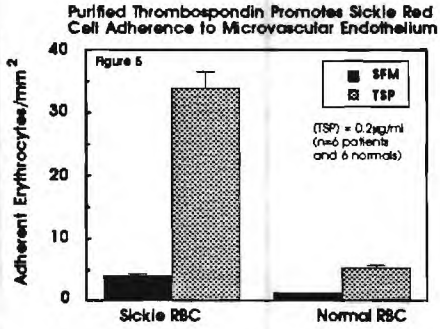


Figure 5. Purified thrombospondin at 0.2 µg/ml concentration promotes higher sickle cell adherence to microvascular endothelium.

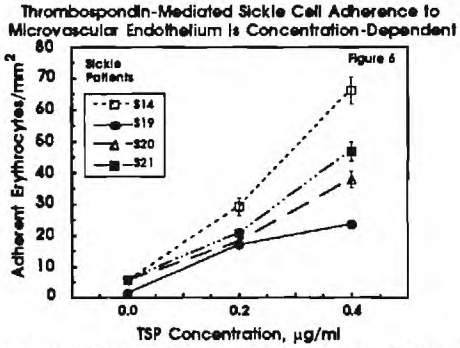
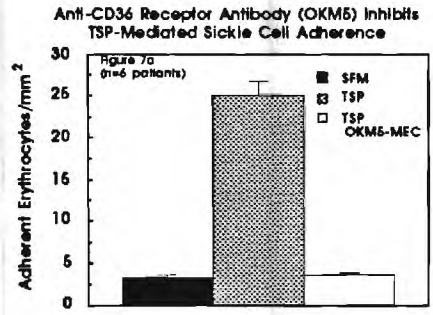


Figure 6. Thrombospondin-mediated sickle cell adherence to microvascular endothelial cells is concentration-dependent.

Thrombospondin binds to endothelial cell CD36 (7) and vitronectin ($\alpha_v\beta_3$) receptors (9). Preincubation of microvascular endothelial cells with anti-CD36 receptor antibody (OKM5, Figure 7a) or anti-α_v antibody (Figure 7b) inhibits thrombospondin-mediated sickle cell adherence by 96% and 99%, respectively.



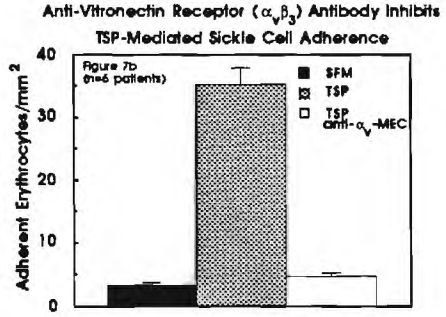
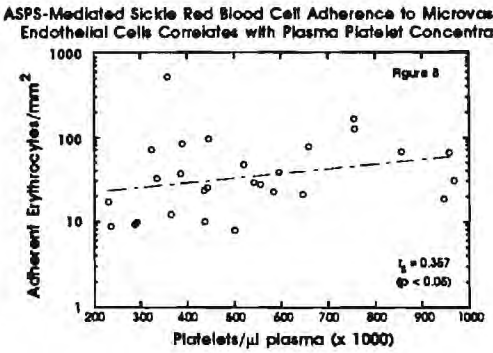


Figure 7. Microvascular endothelial cells were preincubated with OKM5 or anti-α_v antibody for 1 hour prior to the adherence assay. OKM5 or anti-α_v antibody inhibited 99% or 96% of the thrombospondin-mediated adherence, respectively. An antibody against an endothelial cell epitope not thought to be involved in adherence (MHC Class-1) did not inhibit thrombospondin-mediated sickle cell adherence (data not shown).

Finally, for the 28 patients studies to date, the ASPS-mediated adherence levels positively correlate with patient platelet count (Figure 8). These data suggest that platelet-derived adhesion factors may participate in adherence *in vivo*. Additional studies to test this hypothesis are outlined below.



3. Significance

Differences between adherence to microvascular and umbilical vein endothelium.

It is clear from our work (1) and that of others (10) that high molecular weight vWF multimers promote sickle cell adherence to venous endothelium. It is likely that vWF-mediated adherence in the post-capillary venules contributes to vessel occlusion characteristic of sickle cell crisis. However, our new data indicating that plasma, and not high molecular weight vWF, promotes sickle cell adherence to microvascular endothelium (which is predominantly capillary endothelium) suggest that capillary adherence mediated by plasma factors might also contribute to adherence. Thus, further characterization of sickle cell adherence to microvascular endothelium potentially will refine our knowledge of the pathophysiology of vaso-occlusion. Furthermore, the facts that (i) patient-to-patient plasma adherence levels vary widely for asymptomatic patients (see above) and (ii) that for individual patients, plasma-mediated adherence exhibits small longitudinal variation (data not shown) suggests that the level of plasma-mediated adherence might correlate with the variability in clinical complications (frequency and severity) in the sickle population. Obviously, we need additional data to test this hypothesis and this is one goal of the present grant.

Activated Sickle Platelet Supernatant Promotes Sickle Cell Adherence

Many previous investigators have presented data indicating that the coagulation system is activated in sickle patients (see 11 for recent review). These reports have led some to suggest that crisis and microvascular occlusion are, at least in part, thrombotic events. Our new data, presented in Figures 3-7 indicate that factors secreted from activated platelets, including α-granule thrombospondin, promote sickle red cell adherence. Since significant adherence is observed at plasma concentrations of thrombospondin (e.g. 200ng/ml, Fig 5) (8) and thrombospondin concentrations are on the order of 10mg/ml (e.g. 50 times higher) in serum (8), we postulate that thrombospondin-mediated adherence *in vivo* may be very high in the presence of activated platelets. Thus, our new data provide a potential link between platelet activation and microvascular occlusion via adherence of sickle red cells to microvascular endothelium. This exciting hypothesis will drive our experiments for the next budget period.

4. Plans

Specific Aims #1&2. During the next budget period, we plan to explore the following hypothesis related to specific aim #1: Specific plasma factors are responsible for the plasma-mediated adherence to microvascular endothelium observed in Figure 2. We will identifying specific plasma factors (e.g. thrombospondin, fibrinogen, fibronectin, etc.) and endothelial cell receptors (e.g. vitronectin [$\alpha_v\beta_3$], CD36, etc.) which plasma factor(s) bind to by (a) quantifying the level of specific adhesion factors in sickle plasma for each patient, (b) depleting plasma of these factors by affinity chromatography, and (c) utilizing purified protein in experiments similar to those in Figure 5. Additionally, we will identify receptors on endothelial cells which participate in adhesion by blocking access to these receptors with monoclonal antibodies. These studies will be performed essentially as outlined in the funded application.

Specific Aims #2&3. **A major goal** of the next budget period is to further characterize the mechanism of ASPS and thrombospondin-mediated adherence *in vitro*. In addition, we will study patients in

crisis as well as during steady-state to determine whether ASPS or thrombospondin-mediated adherence correlates with patient clinical condition (e.g. crises/year, clinical severity as defined in the literature, etc.). We will also investigate the relationship between CD36 and the vitronectin receptor since antibodies to both essentially block all thrombospondin-mediated adherence. In addition, we will characterize the ASPS-mediated adherence not inhibited by OKM5 (Figure 4). This adherence could be due to other adherence proteins secreted from platelets (e.g. vWF, fibrinogen) or it could be due to endothelial cell stimulation by released ADP, thrombin, or seretonin. *Specific Aim #4*. The endothelial agonists released from activated platelets (e.g. ADP, thrombin, or seretonin) along with cytokines and other vaso-active substances likely upregulate receptor expression of microvascular endothelium in a manner similar to that reported for large vessel endothelium (see 12,13, and original application for full literature review). As outlined in the proposal, modulation of endothelial cell receptor expression is another way to alter sickle cell adherence. These data likely will have clinical relevance as follows. If these physiological mediators (e.g. interferon gamma) increases receptor expression (e.g. CD36, see ref 12) and subsequently elevate sickle cell adherence (the hypothesis we will test), then we can further postulate that *in vivo*, infection (leading to white cell activation and cytokine secretion) could elevate sickle cell adherence to microvascular endothelium and precipitate vaso-occlusion. The fact that infections frequently occur in conjunction with or prior to crisis (14) would appear to make testing our present hypothesis all the more relevant.

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PROGRESS REPORT *(Personnel and Study Subjects)*	GRANT NUMBER HL44960-02

All Personnel for the Current Budget Period
and Any Planned Changes in Personnel for the Next Budget Period

Use two sections. In the first section list *All Current Personnel*. In the second section list *Planned Personnel Changes*.

Name	Degree(s)	SSN	Role on Project *(e.g., PI, Res. Assoc.)*	Date of Birth *(MM/DD/YY)*	Annual % Effort
All Current Personnel					
Timothy M. Wick	B.S., Ph.D.	505-94-2891	PI	07/09/61	25%
James R. Eckman	M.D.	471-48-8946	coinvestigator	08/25/43	5%
Henri A. Brittain	B.S., M.S.	264-53-1153	Grad. Student	01/18/59	100%
Planned Personnel Addition					
Anjali Kumar	B.S.	253-81-0772	Grad. Student	09/15/68	100%

Provide the number of subjects enrolled in the study ***to date*** according to the following categories. (See Page 8 for definitions.)

	American Indian or Alaskan Native	Asian or Pacific Islander	Black, not of Hispanic Origin	Hispanic	White, not of Hispanic Origin	Other or Unknown	TOTAL
Female			33		7		40
Male			40		5		45
Unknown							
TOTAL			73		12		85